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14. ABSTRACT This is the second annual report for the grant to enhance propriospinal relays to bypass a contusion injury to the spinal cord. We have completed experiments for subtask 1 – 4 of specific aim 1 and subtasks 1 for specific aim 2. This Aim 2 of the study, we proposed to examine regeneration and sprouting of supraspinal axons after treatment with our PTEN Antagonist Peptides (1-4). We have previously observed PAP2 to induce robust regeneration in a dorsal hemisection model and thought it might enhance regeneration and sprouting after the more clinically relevant severe contusion injury. Unfortunately, upon completion of the study we did not observe enhanced functional recovery between controls and PAP treated groups. We observed some differences in corticospinal axon tract regeneration and sprouting in the presence of PAP2 compared to PAP1 and PAP4, but not vehicle controls. In addition we observed no statistically significant differences in serotonergic sprouting between controls and PAP treated animals. We are presently starting specific aim 3 of the study to examine enhancing propriospinal axon sprouting using GDNF alone or in combination with NT-3.					
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1. Introduction:

Spinal cord injury causes life-long neurological impairment, with loss of sensory and motor function distal to the point of injury. Despite the clinical impact of traumatic spinal cord injury (SCI), there is no effective treatment for SCI. As a consequence, there are approximately 400,000 patients with SCI in the United States. Experimental therapies to treat SCI have focused on approaches that promote neural regeneration, but major problems remain in achieving long distance regeneration of higher functioning motor control systems, such as the corticospinal tract, making restoration of voluntary locomotor control difficult. In many animal models, spontaneous recovery is often observed after incomplete injuries, leading to partial recovery over time. Spontaneous recovery is thought to be mediated by a number of repair mechanisms including recovery from spinal shock, sprouting and remyelination. Of these processes, sprouting of axons onto interneurons can form adaptive pathways relaying motor information past the lesion to spinal motor neurons driving locomotor responses caudal to the lesion. Indeed several studies using unilateral hemisection model in rodents demonstrate the importance of these relays in supporting spontaneous return of weight support or kinematic patterning of hind limb movements. In several of those studies lesioned supraspinal axons undergo collateral sprouting onto propriospinal interneurons bypassing the lesion. These propriospinal neurons synapse directly onto spinal motor neurons caudal to the lesion site driving locomotor responses. Propriospinal neurons can either span short distances up to six spinal segments or long distances interconnecting cervical and lumbar region. Of particular interest to the return of motor function are the descending propriospinal neurons. The vast majority of these neurons are involved in motor responses and localized to the intermediate zone of the spinal gray matter; an area when stimulated shows locomotor responses. These propriospinal neurons (PN) can either span short distances from the Thoracic to the lumbar region (TPN) or long distances interconnecting cervical and lumbar region. One role of Long-Descending Propriospinal Neuron (LDPN) tracts is to coordinate rhythmic movements of arms and legs during walking. In the cervical cord, they can receive direct supraspinal information from corticospinal, rubrospinal, reticulospinal and tectospinal axons. The majority of these propriospinal axons descend within the medio- and ventro-lateral funiculus entering the ventral horn to form multiple synaptic contacts directly onto motor neurons. After a moderate contusion injury, much of the lateral funiculus is preserved and many PNs are uninjured. The shorter forms of propriospinal neurons are thought to integrate this information, establishing synergistic ensembles to organize movement. Whether or not they contribute to functional recovery after contusive injury has never been determined, and whether or not TPN or LDPNs are more influential in recovery also remains unclear.

2. Keywords: Propriospinal neurons, spinal cord injury, axon sprouting, spinal cord circuits, locomotion, functional recovery.

3. Accomplishments:

Major Task 1: All components of this task have been completed and we are presently finishing the manuscript for submission later this month.

Subtask 1.1: We received ACURO approval to begin animal studies.

Subtask 1.2: We have performed all the histology and we are finishing the stereology on these labeled neurons to determine if there are differences in their numbers between normal and

contused rats. The stereology should have been completed, but we ran into a software problem that took several weeks to solve. We have the majority of this data quantified and should be finished with this part by the end of this month. We are also correlating the changes in neurons bypassing the lesion with the hypothesized behavioral contribution of that pathway. So far, the retrograde labeling correlates well with the observed behavior.

Subtask 1.3: We observed highly levels of genetic retrograde tracer and quantified the changes in neurons before and after injury within multiple locations such as propriospinal neurons; reticulospinal neurons, rubrospinal neurons. We have observed about 30 – 50% of the neurons surviving within these particular regions (this data was presented at the Society of Neuroscience meeting, abst 523.11). This poster is attached to the end of this document.

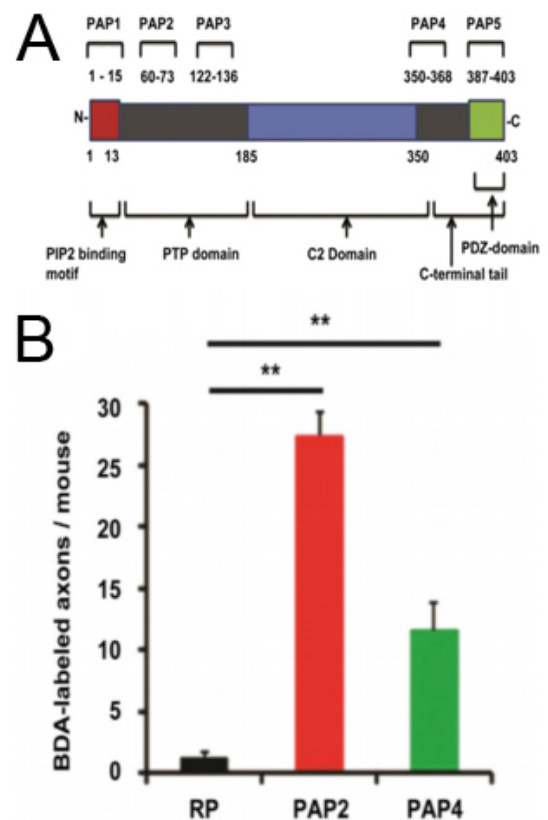
Subtask 1.4: We have performed anterograde tracing and postsynaptic neurons expression studies using AAV coexpressing mCherry and WGA. Although the background was initial high we have improved staining and identified WGA transfer to some propriospinal neurons in the thoracic cord.

Subtask 1.5: We have completed the behavioral studies after neuronal silencing, however the data is presently inconclusive to which surviving neuronal pathway is mediating recovery. It appears that silencing each pathway reduced behavior a small amount but none showed a statistically significant decrease. This may be due to the fact that recovery requires multiple pathways each contributing to locomotion. We will attempt to silence multiple pathways and examine several other behavioral measures to analyze finer details of function for each of the pathways bypassing the lesion.

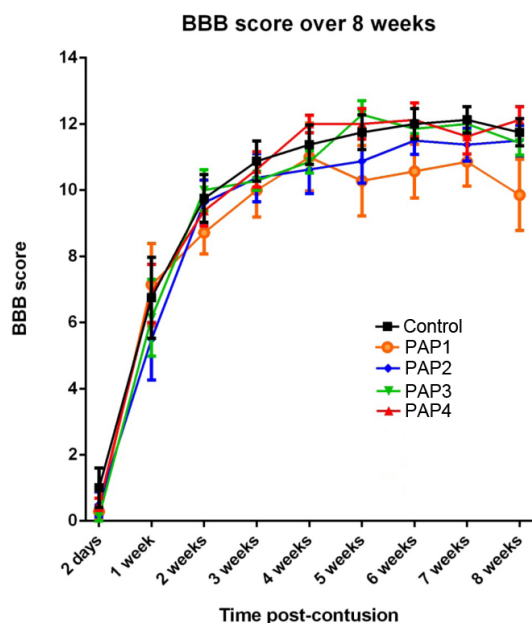
Major Task 2: Examine the contribution of generalized supraspinal sprouting for connectivity onto propriospinal neurons to promote functional recovery.

Subtask 1: Examine the functional contribution of PTEN antagonist-induced supraspinal sprouting for promoting hindlimb locomotion.

Phosphatase and tensin homolog (PTEN) is a negative regulator of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) in cells and functions as a tumor suppressor by inhibiting Akt signaling. Multiple studies in knockout mice have shown the elimination of PTEN greatly increases regeneration and sprouting of retinal ganglion axons and corticospinal axons. We have previously generated four PTEN-Antagonist Peptides (PAPs) to selected regions of the PTEN (Figure 1) that show increased regeneration of corticospinal tract and serotonergic axons after dorsal hemisection, leading to increased functional recovery (Fig 1; Ohtake et al., 2014). In this aim, we proposed to determine if these peptides would increase sprouting using a more clinically relevant T8/9 moderately severe contusion injury model. Our previous annual report (Major Task 1) showed some preservation of reticular spinal pathways and serotonergic axons but not corticospinal pathways. Here the objective was to determine if PTEN antagonist peptides would promote regeneration or sprouting of supraspinal pathways to enhance hindlimb locomotor and



increase functional recovery.

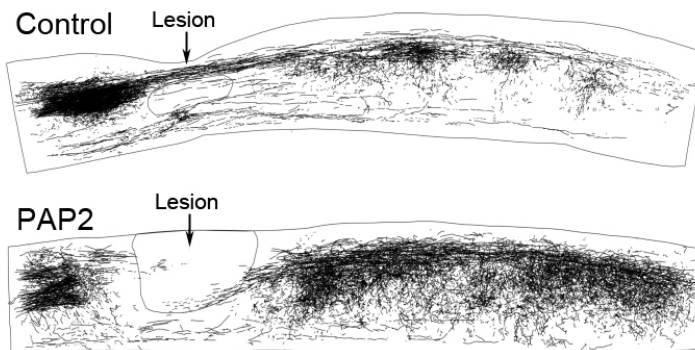
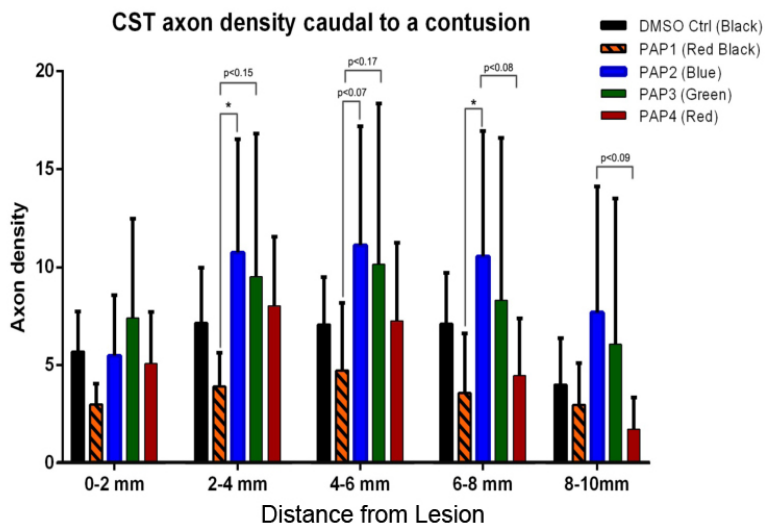


Unfortunately, this treatment regime failed to show statistically significant enhanced functional recovery

as determined by the BBB open field locomotion scores. Weekly assessment showed a very nice spontaneous recovery in all animals; however, there was no statistical significance between control and treatment groups (Fig 2). To examine regeneration of supraspinal axons we analyzed density of labeled corticospinal axons distal to the lesion site. This was performed on every third longitudinal section throughout the entire width of the spinal cord (approximately 70 sections). This was done to make sure the data was analyzed under the most rigorous conditions, particularly since these are spinal cord contusion injuries, which are notoriously variable and often show differential sparing of axons. To quantify axonal regeneration, the number of axons that intersected with a perpendicular line drawn from the dorsal to ventral regions were counted in each section at 1 mm intervals from the lesion site. The data was then

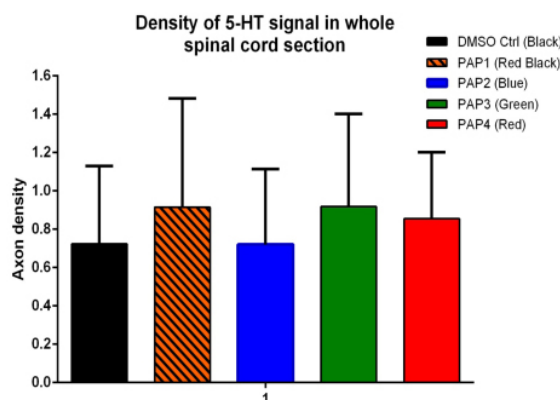
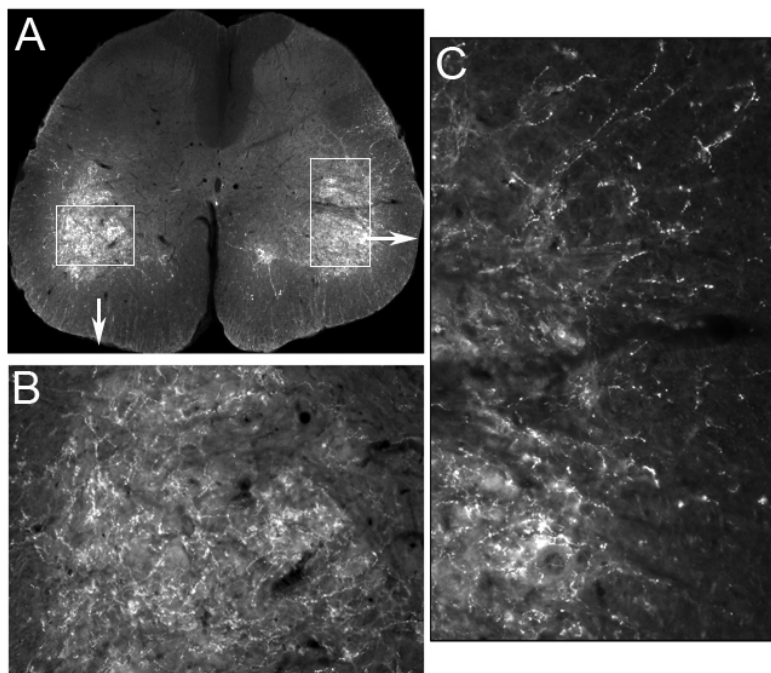
General Methods:

Originally 45 adult rats received T8/T9 contusion injury and randomized into 5 groups (4 peptides and 1 control). Each animal received a 200 kilodyne contusive injury at the thoracic 8/9 level of the dorsal spinal cord, which is considered to produce a severe injury. Unlike our original PTEN antagonist study, which started drug application immediately after injury, this project delayed treatment for 5 days after injury to mimic a more clinically relevant treatment regime. All rats received subcutaneous injections (0.4 mg/rat, two times per day) of either control or PTEN antagonist peptide for 28 successive days. Six weeks after contusion, BDA tracer was injected into the somatomotor cortex and animals euthanized for analysis at 8 weeks post injury. Animals were behaviorally examined using the BBB open field assessment protocol. All data was analyzed blindly and the experimental group code revealed just prior to writing this report.



binned into 2 mm data points for grafting as shown in figure 3. Quantitative data shows that animals treated with PAP2 showed the best growth, but unfortunately it was not significantly different from vehicle control rats. There were significant differences in CST regeneration between rats treated with PAP1 and PAP4, peptides that we previously observed to have a lower effect on regeneration of CST axons (Ohtake et al., 2014). To better appreciate CST labeling in these animal, sections were then combined to generate a composite image of representative spinal cord composites from a vehicle control and PAP2 treated animals are shown (Fig 3 Control or PAP2). Animals treated with PAP2 show increased sprouting of CST axons within the distal cord when compared to controls, however this did not lead to functional recovery.

Since it is well established that regeneration or sprouting of serotonergic (5HT) axons contribute to functional recovery of locomotion, we next quantified the extent of 5HT axons within the lumbar spinal cord, caudal to the lesion site. Serotonergic axons were quantified in cross sections. Although 5HT axons are normally distributed fairly evenly throughout all layers of the spinal cord gray matter, we primarily observed 5HT axons within the ventral horn and very few within the dorsal horn of the spinal cord. Again, the data was relatively disappointing and showed no increase in regeneration or sprouting between control and PTEN peptide treated groups (Fig. 4).



4. Impact:

Unfortunately we are very disappointed by the overall outcome of this study. Although our previous study showed functional recovery of locomotion using the PAP2 PTEN antagonistic peptide after dorsal hemisection lesions, this study with contusive injury failed to repeat those results. This could be due to multiple reasons. One of the major reasons for the difference is mostlike due to contusive injuries being more severe and damaging a larger region of the spinal cord than dorsal hemisection lesions. Unlike hemisections, contusion injuries cause extensive damage to the spinal cord gray and white matter that extends rostral and caudal from the epicenter over several spinal cord segments. In addition, spinal cord contusion injuries cause damage to many more descending supraspinal pathways, particularly those in the ventral region of the spinal cord, than dorsal hemisection. The other difference

between this study and our previous work is the delay in peptide treatment. In the original study we began injecting PTEN antagonistic peptides immediately after injury, here we delayed treatment for 5 days, since this would be a more clinically relevant time point for treatment. The delay may allow the development of a glial-scar to form prior to the onset of axonal regeneration or sprouting; thus acting as a potential barrier to axon growth. Overall this data indicates to us that it might be difficult to induce functional regeneration after spinal cord contusion injury to induce recovery. Indeed, very few studies have observed significant regeneration leading to functional recovery after a more clinically relevant contusion injury. We therefore have begun task 3 to determine if increasing sprouting proximal to the lesion site with induce a relay bypassing the injury.

5. Changes/Problems:

Since we have not observed any functional recovery or statistical significance in regeneration or sprouting with PAP treatment, we have decided to move onto and focus on aim 3. We will not complete task 2 in Aim 2, since it was to assess the levels of synaptic connectivity between CST axons and propriospinal neurons. We think that since this study showed no significant functional recovery or sprouting the examination of synaptic connectivity with this treatment group would not add any information to the overall story. We would like to use the extra time to expand the third aim to include another 2 groups of animals to examine CST sprouting onto propriospinal axons using viral mediated expression of NT3 within propriospinal neurons bypassing the lesion. We consider this to be a potentially interesting experiment for 2 reasons: 1) NT-3 has been shown by multiple groups including ours to induce robust sprouting of corticospinal tract axons and 2) in aim 1 of this study, we showed very good preservation of propriospinal neurons bypassing the contusion lesion. We will use our HiRet lentiviral vector to induce selective expression of NT3 from these propriospinal neurons bypassing the lesion to attract CST sprouting directly onto them. This in combination with the already proposed studies to overexpress GDNF caudal to the lesion will let us identify if sprouting of CST axons onto cervical propriospinal neurons or the sprouting of propriospinal axons distal to the lesion can enhance functional recovery above normal spontaneous recovery levels. We have already made all these virus vectors and tested their function for other studies. We have observed the expression of NT-3 by propriospinal neurons in the cervical region induces robust sprouting of CST axons and increased synaptic connectivity directly onto them. Therefore, this addition will not increase the overall timeline of the project or extra rats, since we will incorporate those original proposed for the synaptic studies from Major Task 2.2.